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Human monoclonal antibody recognizing liver-type aldolase B

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A human hybridoma clone (4E3) has been established by fusing lymphocytes from a lymph node taken from a breast cancer patient and human lymphoblastoid cells, LICR-LON-HMy2, by the poly(ethylene glycol) method. 4E3 has been stabilized and continued to secrete IgM_k antibody into culture medium (> 10 μg/ml) for over 1 year. The following characteristics of the antigen strongly suggested that 4E3 recognizes liver-type aldolase B (EC 4.1.2.13): (i) the M_r of the native molecule is 160000 and that of the subunit is 40000, and thus it has a tetrameric structure of identical subunits; (ii) the antigen is abundant in the liver and kidney of human, mouse and rabbit, and is localized by immunohistochemical methods in the cytoplasm of hepatocytes and in the proximal tubules of the kidney; (iii) the antigen is precipitable by 50–80% saturation with $(NH_4)_2SO_4$; (iv) the antigen shows charge-dependent heterogeneity on DEAE-cellulose chromatography. To confirm this notion, aldolase B was purified to homogeneity from the liver of human, mouse and rabbit by phosphocellulose chromatography. During the chromatographic purification, the antigen activity as assayed by enzyme-linked immunosorbent assay (e.l.i.s.a.) was superimposed on the enzymic activity of aldolase. Furthermore, monoclonal antibody 4E3 strongly reacted with purified aldolase B in SDS/polyacrylamide-gel electrophoresis followed by Western blotting and also in e.l.i.s.a. using microplates coated with purified enzyme. The reaction between aldolase B and 4E3 activated the human complement system as assessed by the attachment of C3 to the immune complex of aldolase B and 4E3.

INTRODUCTION

Most recently in hybridoma technology, much attention and effort have been directed towards human monoclonal antibodies (MAbs) especially for their potential clinical applications. Human hybridomas also will provide a useful tool to analyse the human humoral immune response in autoimmune disease, cancer and allergy (Buck *et al.*, 1984). In an attempt to elucidate the immunological nature of breast-cancer-specific or -associated antigens recognized by patients, we have been preparing human hybridomas using lymphocytes from breast cancer patients. During the course of our experiments, a stable hybridoma clone, 4E3, capable of producing a large quantity of IgM and showing a broad reactivity against various human cancer cells and human tissues was obtained. In immunohistochemical staining, MAb 4E3 reacted strongly not only with normal human but also normal mouse liver and kidney in the cytoplasmic region, suggesting that MAb 4E3 is an autoantibody recognizing some interspecies-common antigen(s). Although several investigators have reported such MAbs both in human and mouse systems (Cote *et al.*, 1983; Houghton *et al.*, 1983; Sikora *et al.*, 1983; Prabhakar *et al.*, 1984), there have been unexpectedly relatively few papers concerning the complete identification or characterization of the antigen molecule itself. In the present study, we provide evidence showing that liver-type aldolase B is the antigen recognized by human MAb 4E3. The biological and immunological significance of the 4E3 clone is discussed.

MATERIALS AND METHODS

Materials

The following reagents were obtained from commercial suppliers: RPMI 1640, foetal calf serum and the mixture of antibiotics were from Gibco, Grand Island, NY, U.S.A.; 50% poly(ethylene glycol) 1450, HAT (50×) and HT (50×) were from BRL, Gaithersburg, MD, U.S.A.; Seaplaque agarose was from FMC, Rockland, ME, U.S.A.; Immunoplate I for e.l.i.s.a. was from Nunc, Roskilde, Denmark; standard human IgG and IgM were from Miles, Elkhart, IN, U.S.A.; rabbit anti-(human Igs) (anti-IgG, anti-IgA and anti-IgM, γ-α- and μ-chain specific respectively) antibodies with and without horseradish peroxidase labelling were from Accurate Chemicals, Westburg, NY, U.S.A.; goat anti-C3 antibody labelled with peroxidase was from Cappel, Cochranville, PA, U.S.A.; DE52 and P11 celluloses were from Whatman, Clifton, NY, U.S.A.; Sephacryl S-300 and Sepharose S-300 and Sepharose 4B were from Pharmacia, Piscataway, NJ, U.S.A.; nitrocellulose membrane and reagents for electrophoresis were from Bio-Rad, Richmond, CA, U.S.A.; aldolase colourimetric assay kit no. 750 was from Sigma, St Louis, MO, U.S.A. All other reagents used were of commercial grade.

Cell lines and cell culture media

The HGPRT$^-$ (hypoxanthine–guanine phosphoribosyltransferase) human lymphoblastoid cell line LICR-LON-HMy2 (HMy2) (Edwards *et al.*, 1982) was kindly

Abbreviations used: Mab, monoclonal antibody; e.l.i.s.a., enzyme-linked immunosorbent assay; PBS, Dulbecco's phosphate-buffered saline; HMy2, human lymphoblastoid cell line LICR-LON-HMy2; HAT, 10^{-4} M-hypoxanthine, 4×10^{-7} M-aminopterin and 1.6×10^{-5} M-thymidine; HT, HAT without aminopterin; BSA, bovine serum albumin.

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provided by the London Branch of the Ludwig Institute for Cancer Research and used as a fusion partner. Various human cancer cell lines (SKBR-3, BxPC-3, HT29, PC3 and FADU) were obtained from American Type Culture Collection (Rockville, MD. U.S.A.). S85 hepatoma cell line was obtained from our colleague, Dr K. Laohathai (Laohathai & Bhamarapravati, 1985). RPMI 640 medium supplemented with 2 mM-glutamine, 10% heat-inactivated foetal calf serum, PSN (penicillin/streptomycin/neomycin) antibiotics and gentamycin (50 μg/ml), referred to as complete medium, was used for growing HMy2, human cancer cell lines and stabilized human hybridoma cells. Before stable hybridoma clones were developed, the concentration of foetal calf serum was increased to 15% and 50 μM-2-mercaptoethanol was added to the medium. The complete media with 15% foetal calf serum and 50 μM-2-mercaptoethanol supplemented with HAT and HT are referred to as HAT medium and HT medium, respectively. Hybridoma cells and various human cancer cell lines were maintained at 37 °C in humidified air containing 5% CO_2.

Preparation of cells from lymph nodes

Segments of axillary lymph nodes taken from patients with histologically confirmed breast carcinoma were processed aseptically within 2 h of surgery. Excess fat was removed from the nodes, and single-cell suspensions were prepared by mincing and teasing the tissue with surgical scissors and forceps. The cell suspensions were washed twice with serum-free RPMI 1640 medium by centrifugation at 500 *g* for 5 min.

Cell fusion and cloning

Cells from lymph nodes and human lymphoblastoid cells, HMy2, in exponential phase of growth were mixed in a centrifuge tube (Falcon 2070) at a ratio of 2:1, centrifuged and fused by using the poly(ethylene glycol) method (Galfre *et al.*, 1977) with some modifications. Poly(ethylene glycol) 1450 (1 ml of 50%) prewarmed at 37 °C was added dropwise for 1 min to the cell pellet mixture. During this period and for an additional 30 s, the centrifuge tube was rotated gently and slowly by hand. Poly(ethylene glycol) was then diluted during a 3-min period with serum-free medium in the following way: 1 ml, 2 ml and 4 ml were added successively at 1 min intervals, and finally 30 ml was added. The post-fusion product was centrifuged at 500 *g* for 5 min and resuspended in complete medium with foetal calf serum and 2-mercaptoethanol (3×10^6 cells/ml). Cell suspensions (100 μl) were plated into each well of 96-well tissue culture plates (Falcon 3072), and 24 h later 100 μl of HAT medium was added to each well. At 2 day intervals, 100 μl of spent medium were replaced by an equal volume of HAT medium. This procedure was repeated for 2–3 weeks until the growth of hybrid colonies reached 20–30% confluency. Supernatants from each well with formation of such colonies were assayed for production of human Igs and also for the reactivities with various human cancer cell lines as described below. Positive hybrid cells were transferred to 24-well plates (Falcon 3047) and expanded with HT medium. The hybrid cells were then cloned by the soft-agar method (Kennet, 1980). The cells were counted and diluted to allow 500 and 5000 cells to be seeded respectively into centrifuge tubes (Falcon 2095) containing 8 ml of 0.3% agarose dissolved at 42 °C in the complete medium with foetal calf serum and 2-mercaptoethanol. After solidification of the agarose in an ice bath, the tubes were incubated at 37 °C for 2–3 weeks until macroscopic colonies appeared. Single colonies were picked up from the agarose with a Pasteur pipette and cultured in 24-well plates. When clones had grown to 30–40% confluency, supernatant from each well was again assayed for the concentration and activity of MAb. Repeated cloning was done to select more stable and high Ig-producing hybrid cells.

Human antibody screening assay

Human Igs by e.l.i.s.a. Spent media from wells exhibiting the growth of hybrid cells were assayed for the concentration of human Igs or IgM by e.l.i.s.a. (Voller *et al.*, 1976). Anti-Igs or anti-μ antibody dissolved in 0.1 M-$NaHCO_3$ (100 μl, 50 μg/ml) were added to each well of an Immunoplate I and incubated for 1 h at room temperature. The wells were then replaced with 100 μl of 1% BSA dissolved in PBS containing 0.01% thiomersal (referred to as 1% BSA) and incubated for 1 h at room temperature to reduce nonspecific adsorption of proteins during the assay. Each well was washed once with PBS and subjected to the assay. Spent media (100 μl diluted 1:5 with 1% BSA) were added to the antibody-coated wells in duplicate and incubated at 37 °C for 2 h. Standards of human IgG and IgM, ranging from 1 to 1000 ng/ml, were included for each assay. The wells were then washed three times with PBS, and 100 μl of horse-radish peroxidase-labelled anti-Igs or anti-μ antibody diluted 1:2500 with 1% BSA were added to each well. The plate was incubated at 37 °C for 2 h followed by washing three times with PBS. Substrate solution (100 μl of 16 mM *o*-phenylenediamine/0.02% H_2O_2 in 0.1 M-citric acid/0.2 M-Na_2HPO_4 buffer, pH 5.5, containing 0.01% thiomersal) was added to each well, and the reaction was continued for 10–30 min at room temperature. The enzyme reaction was terminated by adding 100 μl of 1 M-HCl, and the absorbance was read at 488 nm in an e.l.i.s.a. reader (Fisher Scientific Co.).

Reactivity with human cancer cell lines by e.l.i.s.a. This screen test was performed using various human cancer cells grown and fixed to 96-well culture plates (Falcon 3072) as target cells (Capone *et al.*, 1984). Each cancer cell line in exponential phase of growth was treated with 0.25% trypsin/0.025% EDTA in PBS to make a single-cell suspension. The cells were washed once and resuspended in complete medium (5×10^5 cells/ml). Cell suspensions (200 μl) were seeded into each well of 96-well plates and incubated at 37 °C for 1–5 days until the growth of cells reached confluency. The cells grown in each well were washed three times with PBS and then fixed sequentially with 100 μl each of 2% formalin/3% sucrose in PBS (10 min), 0.5% Triton X-100 in 20 mM-Hepes buffer, pH 7.4, containing 0.3 M-sucrose, 3 mM-$MgCl_2$ and 0.5 M-NaCl (10 min), and 2% formalin/3% sucrose in PBS (10 min) (Nigg *et al.*, 1983). After washing three times with PBS, each well was filled with 100 μl of 1% BSA in PBS containing 0.1% NaN_3 and stored in a humidified chamber at 4 °C until use. The screening assay of hybridoma spent media was carried out in the same way as described in the previous section, except that peroxidase-labelled anti-Igs or anti-μ antibody diluted 1:1000 were used as the second antibody.

Production of ascites fluid in nude mice and purification of IgM from ascites

Human hybridoma cells (2×10^7) were injected intraperitoneally into Balb/c athymic nude mice which had been primed 1 and 7 days earlier with 0.5 ml of pristane (Abrams *et al.*, 1984). At 2–4 weeks later, about half of the mice produced ascites. The ascites was collected through an injection needle, centrifuged at 10000 *g* for 10 min and stored at −70 °C until use. The purification of IgM from ascites was performed by using an immunoaffinity column of anti-μ antibody. Anti-μ antibody (25 mg) was bound to 5 ml of Sepharose 4B by the CNBr method (Porath *et al.*, 1973). The ascites was fractionated twice with 50% saturated $(NH_4)_2SO_4$ and centrifuged at 10000 *g* for 10 min. The precipitate was disssolved and dialysed against PBS. The resultant material was applied to the anti-μ–Sepharose 4B column (1 cm × 7 cm) equilibrated with PBS. The column was washed extensively with PBS and IgM was eluted with 3 M-KSCN. The absorbance at 280 mn was determined and fractions exhibiting absorbance were dialysed immediately against PBS, concentrated and stored at −70 °C. The amount of IgM in ascites and fractions from the column was determined by the classical Mancini test or by e.l.i.s.a. as described above. The purified IgM MAb was used in elucidating the specific reaction between MAb and the antigen it recognized.

Immunoperoxidase staining

Sections (4 μm thick) of Bouin's fluid-fixed, paraffin-embedded tissues were deparaffinized and incubated with 0.6% H_2O_2 in methanol to block endogenous peroxidase activity. After rinsing in PBS, the slides were incubated with 10% normal mouse serum for 10 min. The serum was removed and nude mouse ascites diluted with 1% normal mouse serum (100 μg of MAB/ml) or polyclonal human IgM (used at an identical IgM concentration) as a negative control was added for 2 h. The slides were then rinsed for 3 × 5 min in PBS and incubated with a 1:100 dilution of peroxidase-labelled anti-μ antibody in 1% normal mouse serum for 1 h. The slides were then rinsed as above, and allowed to react with 0.02% diaminobenzidine/0.03% H_2O_2 in 0.05 M-Tris/HCl, pH 7.7, for 5–20 min. After rinsing in PBS, the slides were counterstained with haematoxylin, dehydrated and mounted with glycerol/gelatin.

Preparation of tissue extracts and subcellular fractionation

Normal human and mouse tissues were minced in 4 vol. of 10 mM-Tris/HCl, pH 7.2, containing 0.5% Nonidet P-40 and 2 mM-phenylmethanesulphonyl fluoride (20% wet w/v) and homogenized at 0 °C for 5 min with a Polytron homogenizer (Brinkman, Westburg, NY, U.S.A.). The homogenates were clarified by centrifugation at 10000 *g* for 10 min, and the supernatants were used for further experiments. As for subcellular fractionation of normal mouse liver, the method described by Hogeboom (1955) was employed. Briefly, 2 g of mouse liver was homogenized in 18 ml of 0.25 M-sucrose with a Potter–Elvehjem homogenizer, overlayed on 20 ml of 0.34 M-sucrose and centrifuged at 700 *g* for 10 min (precipitate and supernatant are referred to as P1 and S1, respectively). P1 is the nuclear fraction. S1 was centrifuged at 5000 *g* for 10 min to separate P2 and S2. P2 was washed with 10 ml of 0.25 M-sucrose by centrifugation at 24000 *g* for 10 min (P3 and S3). P3 was washed once more in the same way as above (P4 and S4); P4 is the mitochondrial fraction. S2, S3 and S4 were pooled, made up to 70 ml and centrifuged at 54000 *g* for 60 min (P5 and S5). P5 and S5 are the microsomal and supernatant fractions, respectively. The nuclear, mitochondrial and microsomal fractions are disrupted by sonication (Fisher Scientific instrument) in 4 ml of 10 mM-Tris/HCl, pH 7.2, containing 0.5% Nonidet P-40 and 2 mM-phenylmethanesulphonyl fluoride and centrifuged at 10000 *g* for 10 min. The resultant supernatants were used for experiments.

Partial purification of antigen(s)

Liver or kidney homogenate (20 ml) from normal human and mouse as prepared above was fractionated at 0 °C with 45–80% saturated $(NH_4)_2SO_4$. The precipitates were dissolved and dialysed at 4 °C against PBS. The dialysates were clarified by centrifugation at 10000 *g* for 10 min and applied to a Sephacryl S-300 column (2.5 cm × 95 cm) equilibrated with PBS. Elution was performed at a flow rate of 10 ml/h with PBS (10 ml/tube). The active antigen fractions were pooled, concentrated and dialysed against 20 mM-sodium phosphate buffer, pH 8.0 (pH 7.2 for mouse kidney). These pooled fractions were then subjected to chromatography on a DE52 DEAE-cellulose column (1.4 cm × 20 cm) equilibrated with the same buffer, and eluted at a flow rate of 10 ml/h with 200 ml of a linear gradient of NaCl up to 0.2 M (0.4 M for mouse kidney). The assay for the antigen(s) was performed by two different methods as described below under 'Assay of antigen'.

Purification of liver adolase B

This was carried out according to an established procedure (Penhoet *et al.*, 1969) using, as starting material, human, mouse and rabbit livers (50 g, 25 g and 45 g, respectively). The purified aldolase was dialysed against 10 mM-Tris/HCl (pH 7.5) containing 1 mM-EDTA, concentrated and stored at −70 °C.

Assay of aldolase activity

Enzyme activity was determined using the aldolase colourimetric assay kit no. 750 (Sigma) according to the manufacturer's instructions.

SDS/polyacrylamide-gel electrophoresis

Electrophoresis was carried out using slab-type gradient polyacrylamide gels (4–18%; 17 cm × 13 cm × 0.15 cm) according to the method of Laemmli (1970) using a 3% stacking gel. The electrophoresis was run for 16 h at a constant current of 8 mA. Proteins in gels were stained for 30 min with 0.25% Coomassie Brilliant Blue R250 in 50% methanol/7% acetic acid, and destained overnight in 10% methanol/5% acetic acid.

Assay of antigen

E.l.i.s.a. Sample fractions to be tested were diluted appropriately with 0.1 M-$NaHCO_3$ (usually 1:20) and 100 μl of each was coated at room temperature for 1 h onto a 96-well Immunoplate I. The wells were washed once with PBS, and incubated at room temperature for 1 h with 100 μl of 1% BSA to eliminate or reduce non-specific binding. After washing once more with PBS,

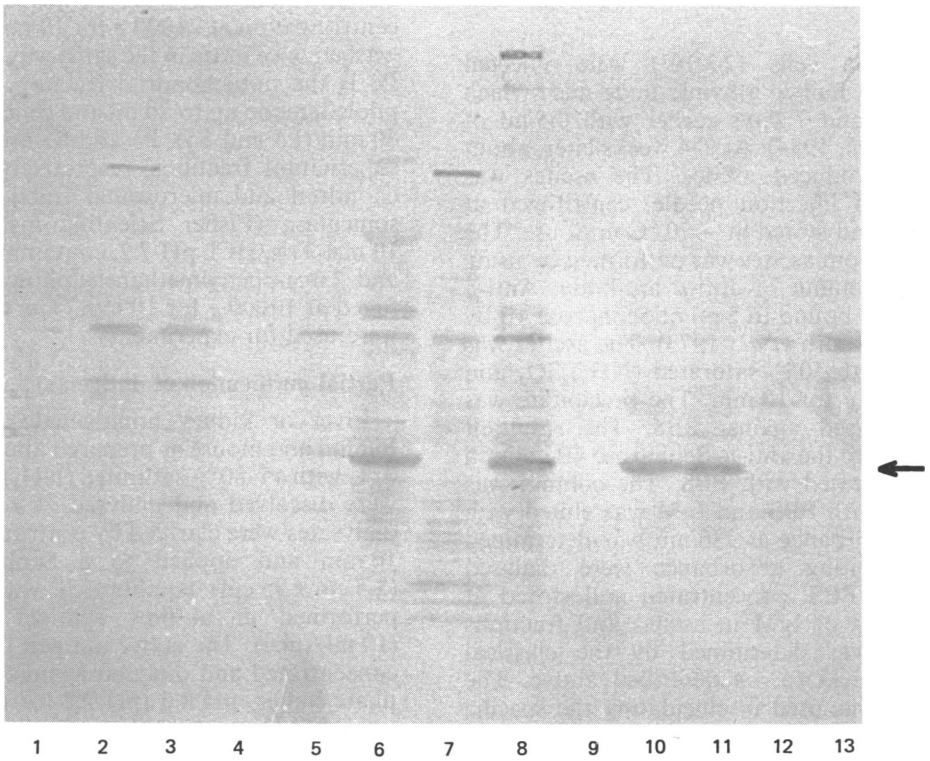

Fig. 1. Tissue distribution of antigen recognized by MAb 4E3 in Western blotting analysis

Extracts from various normal human and mouse tissues prepared as described in the Material and methods section were separated by SDS/polyacrylamide-gel electrophoresis on 4–18% polyacrylamide gels. After transferring to nitrocellulose membrane the antigen, indicated by the arrow, was visualized by using immunoblotting technique. Lanes 1–9 are tissue extracts from mouse brain (lane 1), lung (lane 2), heart (lane 3), stomach (lane 4), muscle (lane 5), liver (lane 6), spleen (lane 7), kidney (lane 8) and intestine (lane 9). Lanes 10–12 are those from human liver (lane 10), kidney (lane 11) and spleen (lane 12). Lane 13 is human IgM (Miles Co.) as reference. Note that the band corresponding to M_r 40000 is strongly stained in the liver and kidney preparations from both human and mouse.

wells were incubated at 37 °C for 2 h with 100 μl of MAb spent media (2–10 μg of IgM/ml), and the plate was washed three times with PBS. In anti-C3 e.l.i.s.a., each well was treated at 37 °C for 30 min with normal human serum diluted 1:120 as a source of complement, washed once with PBS and incubated at 37 °C for 2 h with peroxidase-labelled anti-C3 antibody (1:2500 dilution). After washing three times with PBS, peroxidase activity was assayed as described above.

Western blotting. Proteins separated by SDS/polyacrylamide-gel electrophoresis were transferred to a nitrocellulose membrane at 50 V for 3 h in 25 mM-Tris/192 mM-glycine, pH 8.3, containing 20% methanol according to the method of Towbin *et al.* (1979). After transfer, the nitrocellulose membrane was incubated at 37 °C for 1 h in 5% BSA in PBS containing 0.05% Nonidet P-40 and 0.01% thiomersal (referred to as 5% BSA/NP-40) to block nonspecific binding. The nitrocellulose membrane was then incubated with MAb (5 μg of IgM/ml) followed by horseradish peroxidase-labelled anti-μ antibody (1:500 dilution). These antibodies were diluted with 5% BSA-NP-40 and incubation of each antibody was for 1.5 h at room temperature. After incubation with each antibody, the nitrocellulose membrane was extensively washed twice with PBS containing 0.05% Nonidet P-40 and once with PBS (10 min each). The Western blots were stained for 5–20 min with saturated diaminobenzidine in 0.1 M-citric acid/0.2 M-NaH_2PO_4, pH 5.5, containing 0.03% H_2O_2 and 0.01% thiomersal, and reaction was stopped with 7% acetic acid.

RESULTS

Generation of human MAb 4E3

A human hybridoma cell producing IgM-type MAb (4E3) has been established in one of the fusion experiments using the lymphocytes from a breast cancer patient and the human lymphoblastoid cells, HMy2. After fusion, the resulting cells were seeded into 490 wells of 96-well culture plate, and colonies developed in 133 wells. The number of wells producing human IgM at over 100 ng/ml was 25, and only one of these 25 hybrids, designated 4E3, has continuously secreted a large amount of human IgM into culture medium (> 100 μg/ml). 4E3 also exhibited reactivity against various human cancer cell lines as described below. Although the production of IgM decreased gradually, 4E3 has been stably producing 10–20 μg of IgM/ml for over 1 year, upon repeated cloning with semi-solid agarose. Ouchterlony test revealed that the antibody is of IgM_κ isotype and the antibody purified with immunoaffinity column showed two bands, μ chain (70 kDa) and

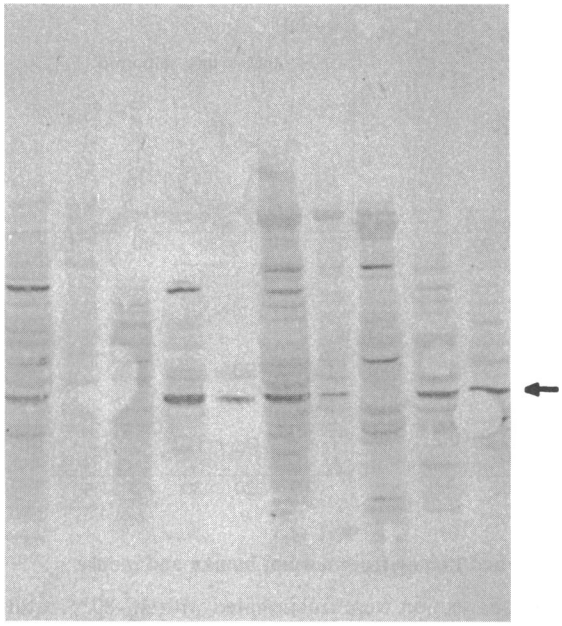

Fig. 2. Detection of the antigen recognized by MAb 4E3 in the subfraction of mouse liver and kidney and by Western blotting technique

Extract from normal mouse kidney was precipitated with various concentrations of $(NH_4)_2SO_4$, and that from normal mouse liver was separated into its subcellular components as described in the Materials and methods section. The antigen in these samples (arrow) was visualized by Western blotting analysis. Lanes 1–4 are from mouse kidney, whole tissue extract (lane 1) and fractions precipitated with 0–33% (lane 2), 33–50% (lane 3) and 50–80% (lane 4) saturated $(NH_4)_2SO_4$, respectively. Lane 5 is partially purified antigen from mouse kidney on Sephacryl S-300 column (refer to Fig. 3*a*). Lanes 6–10 are from mouse liver, whole tissue extract (lane 6), nuclear (lane 7), mitochondrial (lane 8), microsomal (lane 9) and supernatant (lane 10) fractions, respectively. Note that the antigen is precipitated between 50 and 80% $(NH_4)_2SO_4$ saturation (lane 4), and recovered in microsomal and supernatant fractions (lanes 9 and 10).

light chain (23 kDa) on SDS/polyacrylamide-gel electrophoresis. Approximately one-half of the athymic nude mice injected with 4E3 hybridoma produced ascites which contained 0.1–1 mg of IgM MAb/ml.

Reactivity of MAb 4E3 against cancer cell lines

In cell e.l.i.s.a. using human cancer cell lines as target antigen(s), MAb 4E3 reacted with a broad spectrum of tumour cells including SKBR-3 (breast), BxPC-3 (pancreas), HT29 (colon), S85 (liver), PC3 (prostate), and FADU (pharynx), with various degrees of intensity [in the order PC3 (= 100) > HT29 (82) > SKBR-3 (71) > BxPC-3 (68) > S85 (39) > FADU (32)]. This finding suggested that 4E3 reacted with normal or some widely distributed components in cultured cells. A stronger reaction of MAb 4E3 with each cell line was detected after treatment of formalin-fixed cells with Triton X-100, suggesting that MAb 4E3 recognized cytoplasmic antigen(s) or internal components of the membrane.

Characterization of antigen(s)

Immunohistochemical analysis. The distribution of antigen(s) recognized by MAb 4E3 on paraffin-embedded sections prepared from a variety of normal and cancerous human tissue was examined by indirect immunoperoxidase staining. Positive reaction was evident in cancer tissues examined, including breast, kidney, liver, colon and prostate. However, normal liver and kidney were stained much more strongly with 4E3. The cytoplasm of almost all the hepatocytes was stained most intensively. In sections of the normal kidney specimen, a characteristic pattern of intense staining was detected in the proximal tubules. Human renal cell carcinoma showed a mixed pattern of both strong and weak staining. However, normal spleen and most of the blood cells were stained negatively. These findings suggested that the antigen(s) was a ubiquitous normal cell component(s) and most abundant in normal human liver and kidney. Furthermore, normal mouse liver and kidney were also stained strongly with MAb 4E3 in a similar pattern to those of human tissues, suggesting that 4E3 recognized some interspecies-common antigen(s).

SDS gel electrophoresis and Western blot analysis. In order to identify the antigen(s) recognized by MAb 4E3, various tissue extracts from both human and mouse were prepared and analysed for antigen molecule(s) by SDS/polyacrylamide-gel electrophoresis followed by Western blotting. As shown in Fig. 1, the band corresponding to M_r 40000 was strongly stained in the liver and kidney preparations from both human and mouse. A couple of other bands also were stained positively with normal human IgM in place of MAb 4E3, indicating that they were caused by non-specific binding of peroxidase-labelled anti-μ antibody used as the second antibody. The tissue distribution of antigen analysed by Western blotting was in good agreement with the histochemical studies as described above. The electrophoretic mobility of the M_r 40000 antigen was not changed by the presence or absence of 2-mercaptoethanol.

Subcellular distribution. In some hepatic diseases, autoantibodies to subcellular components of liver have been reported. These are, for instance, anti-(liver/kidney microsome) antibody in children with autoimmune chronic active hepatitis (Alvarez *et al.*, 1985) and anti-mitochondrial autoantibody in primary biliary cirrhosis (Frazer *et al.*, 1985). In light of these reports a simple subcellular fractionation was performed using freshly obtained normal mouse liver. As shown in Fig. 2, the M_r 40000 antigen was detected in both microsomal and postmicrosomal (supernatant) fractions by Western blotting, supporting our immunohistochemical observations that the antigen was stained in the cytoplasmic region. The faint band in nuclear fractions may be attributed to contamination by undisrupted cells.

Partial purification of M_r 40000 antigen. High antigen content and its restricted immunoperoxidase staining in the proximal tubules led us to select normal human or mouse kidney as a starting material for initial purification of the putative M_r 40000 antigen. At first, the precipitability of the antigen with $(NH_4)_2SO_4$ was examined using normal mouse kidney extract. The 20% tissue homogenate preparation (wet w/v) was precipita-

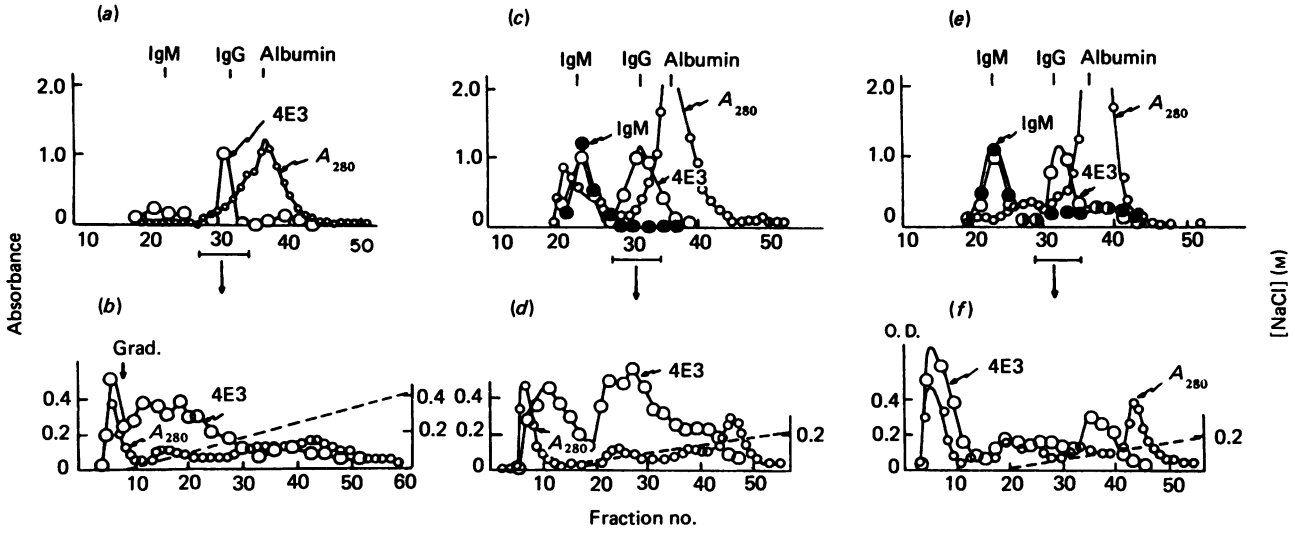

Fig. 3. Partial purification of the antigen recognized by MAb 4E3 from liver and kidney from normal human and mouse

Tissue extracts (20 ml) prepared as described in the Materials and methods section were fractionated with 45–80% saturated $(NH_4)_2SO_4$ and then applied to a Sephacryl S-300 column (2.5 cm × 95 cm) equilibrated with PBS (panels *a*, *c* and *e*). Fractions (10 ml) were collected and analysed for the presence of the antigen by anti-μ e.l.i.s.a. Active fractions, indicated by the horizontal line, were pooled, concentrated and dialysed against 0.02 M-sodium phosphate buffer, pH 8.0 (pH 7.5 for mouse kidney). The resultant samples were applied to a DEAE-cellulose column (1.4 cm × 20 cm) equilibrated with the same buffer (panels *b*, *d* and *f*). Elution was performed with 200 ml of a linear gradient (Grad.) of NaCl up to 0.2 M (0.4 M for mouse kidney). Fractions (5 ml) were collected and assayed for antigen activity by anti-μ e.l.i.s.a. Note that the antigen was eluted at almost the same position as human IgG from the Sephacryl S-300 column, and showed heterogeneity on the DEAE-cellulose column. (*a*) and (*b*), mouse kidney; (*c*) and (*d*), human kidney; (*e*) and (*f*), human liver.

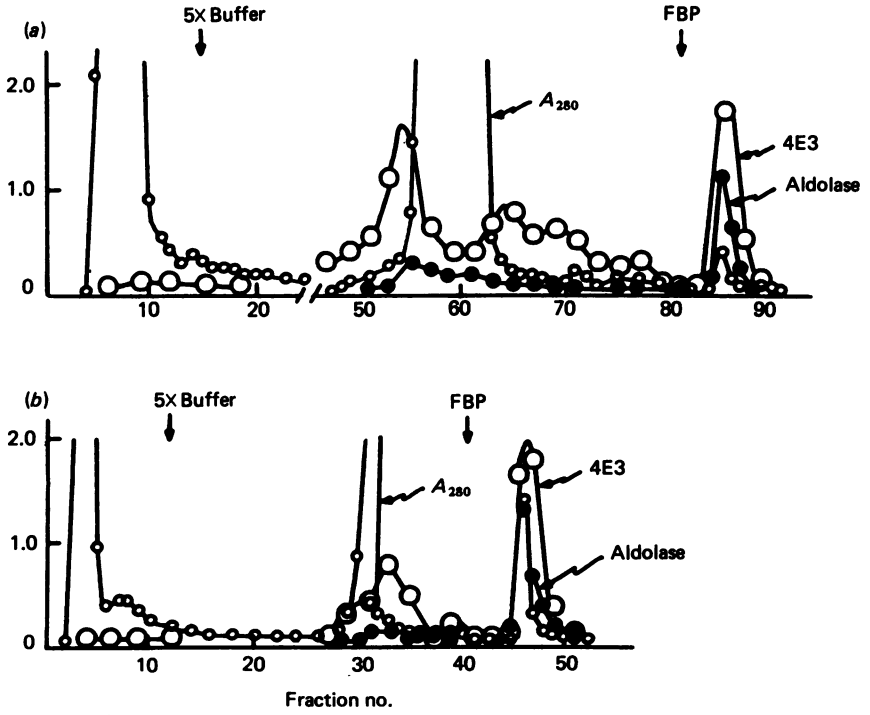

Fig. 4. Purification of aldolase B from human and mouse livers by phosphocellulose chromatography

Human (*a*) and mouse (*b*) liver extracts prepared as described in the Materials and methods section were applied to phosphocellulose columns (2.5 cm × 15 cm for human liver and 1.4 cm × 20 cm for mouse liver) which had been equilibrated with 10 mM-Tris/HCl, pH 7.5, containing 1 mM-EDTA (TE buffer). After extensive washing with 5 × TE buffer, aldolase was eluted with 2.5 mM-fructose 1,6-bisphosphate (FBP). Fractions (10 ml) were assayed both for the presence of antigen by anti-μ e.l.i.s.a. (4E3) and for enzyme activity of aldolase with an assay kit by measuring the colour intensity of the product at 750 nm. Enzyme activity with $A = 1$ was equivalent to approx. 1000 units/ml. Note that the activity of antigen as detected by MAb 4E3 was in good accordance with enzymic activity of aldolase in the fractions eluted with 2.5 mM-fructose bisphosphate.

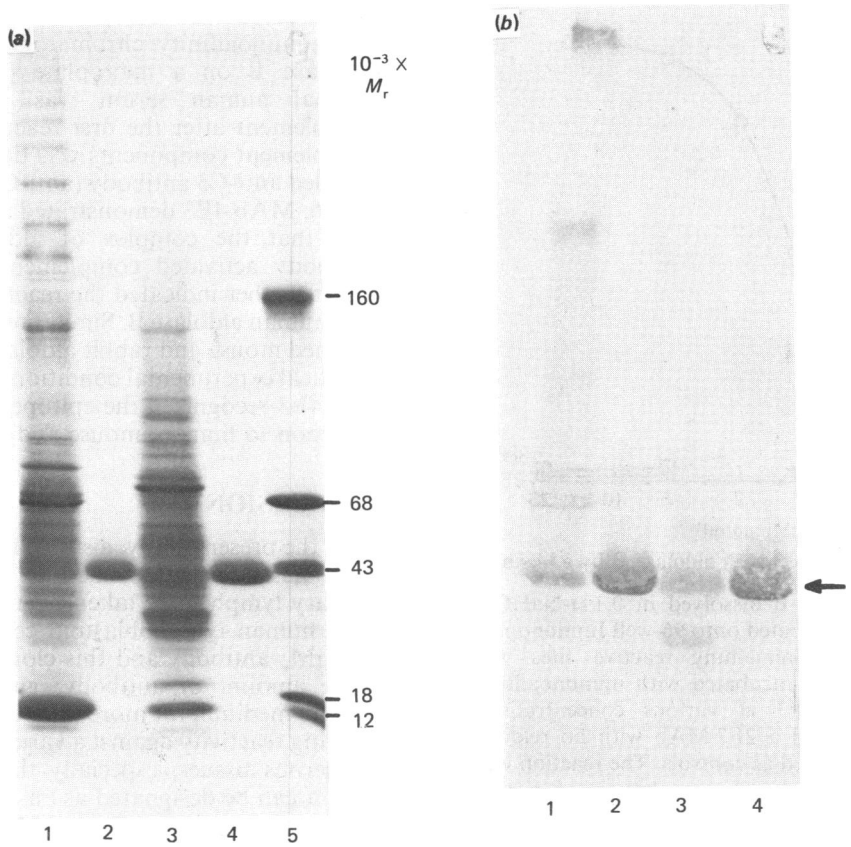

Fig. 5. SDS/polyacrylamide-gel electrophoresis of purified human and mouse aldolase B and its immunoblotting with MAb 4E3

Aldolase purified from human and mouse livers by phosphocellulose chromatography was separated by SDS/polyacrylamide-gel electrophoresis on 4–18% polyacrylamide gels, and subjected to Western blotting with MAb 4E3. Lanes 1 and 3 are human and mouse liver homogenates, and lanes 2 and 4 are purified aldolase B from human and mouse liver, respectively. Lane 5 is M_r markers. Panel (*a*) is protein staining and panel (*b*) is Western blotting with MAb 4E3. Note that bands of both human and mouse aldolase B are strongly stained with MAb 4E3 and the same position of both lanes 1 and 3 in (*b*) is also stained.

ted sequentially with 33%, 50% and 80% saturated $(NH_4)_2SO_4$ and the presence of antigen molecule in these fractions was analysed by Western blotting. As shown in Fig. 3, most of the M_r 40000 antigen was precipitated between 50 and 80% saturation with $(NH_4)_2SO_4$. With this initial observation, mouse kidney extract was first precipitated with 50–80% $(NH_4)_2SO_4$ and the resulting dialysed fraction was then chromatographed on a Sephacryl S-300 column. As shown in Fig. 3(*a*), the MAb 4E3 reactive peak assayed by e.l.i.s.a. was eluted at almost the same position as the human IgG marker from the column (fractions 27–34), indicating that the native M_r of the antigen was around 16000. By SDS/polyacrylamide-gel electrophoresis and Western blotting, the M_r 40000 band was stained in good accordance with the active fractions (fractions 27–34). These results suggested that the native molecule of M_r 40000 antigen was composed of four similar subunits (thus a tetramer). The active fractions were pooled and then subjected to DEAE-cellulose chromatography. As shown in Fig. 3(*b*), the MAb 4E3-reactive fractions were split into several peaks and the M_r 40000 band analysed by Western blotting was observed in every peak (results not shown), suggesting that the antigen possessed charge-dependent heterogeneity. The chromatographic profiles of MAb 4E3-reactive antigen from human kidney on Sephacryl S-300 and DEAE-cellulose columns were almost identical with those of mouse kidney (Figs. 3*c* and 3*d*), indicating that the M_r 40000 antigen from these two specimens was identical or quite similar. The apparent active peak eluted after the void volume in human kidney extract (fractions 21–23) was attributed to IgM in the extract to which peroxidase-labelled anti-μ antibody used as the second antibody was bound directly, since the same reaction was also seen with human IgM instead of MAb 4E3 and no corresponding active peak was seen in mouse kidney extract (Fig. 3*a*). Additional chromatographic profiles of MAb 4E3-reactive antigen in human liver preparation as shown in Figs. 3(*e*) and 3(*f*) revealed almost identical patterns to those of human and mouse kidney preparations, indicating that these two different tissues contained the same or quite similar antigen molecule.

Deductive identification of the antigen. The results obtained above strongly suggested that the antigen as recognized by MAb 4E3 is most likely a liver-type aldolase B, one of three aldolase isoenzymes A, B and C. Namely: (i) it is abundant in cytoplasm of the liver and proximal tubules of the kidney, and absent from the spleen and most of blood cells (Lebherz & Rutter, 1969); (ii) it is an interspecies-common component (Lebherz & Rutter, 1969); (iii) it is precipitable with 50–80% saturated $(NH_4)_2SO_4$ (Penhoet *et al.*, 1969); (iv) the M_r

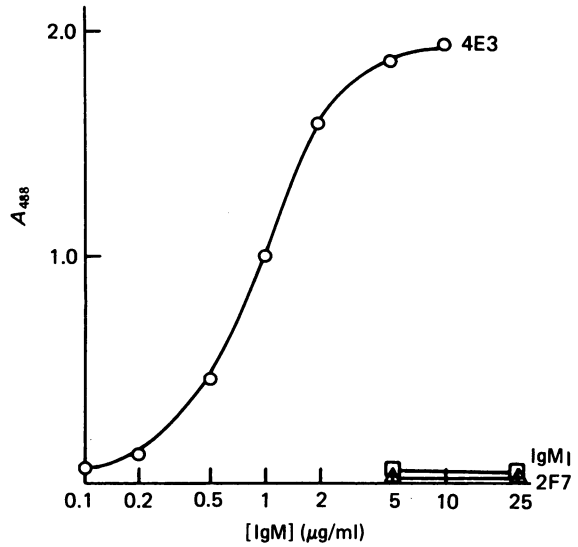

Fig. 6. Reactivity of MAb 4E3 with aldolase B by e.l.i.s.a.

Purified human aldolase B dissolved in 0.1 M-$NaHCO_3$ (100 μl of 5 μg/ml) was coated onto 96-well Immunoplate I. After blocking the remaining reactive sites with 1% BSA, each well was incubated with immunoaffinity column-purified MAb 4E3 at various concentrations. Normal human IgM and a 2F7 MAb with no relevant antibody activity were used as controls. The reaction with MAb 4E3 was followed by the incubation of normal human serum as complement, and peroxidase-labelled anti-C3 antibody was used as detection reagent. In these experiments, MAb 4E3 did not show any reactivity when the microplate was coated with proteins other than aldolase B, such as BSA, transferrin and lactoferrin (results not shown). See the Materials and methods section for experimental procedures in detail.

of the native molecule is 160000 and that of the subunit is 40000, and thus it has a tetrameric structure of identical subunits (Gracey *et al.*, 1969); (v) it possesses charge-dependent heterogeneity (Blostein & Rutter, 1963). To confirm this working hypothesis, aldolase B was purified from human, mouse and rabbit livers by a previously established method (Penhoet *et al.*, 1969). Fig. 4 shows the elution profile of the enzyme from human and mouse liver extracts from a phosphocellulose column. In the fractions eluted with 2.5 mM-fructose bisphosphate (fractions 85–89 for human and 46–49 for mouse) which contained highly purified aldolase B as judged by SDS/polyacrylamide-gel electrophoresis (Fig. 5), antigen activity by e.l.i.s.a. was superimposed on the enzymic activity of aldolase. Furthermore, MAb 4E3 reacted strongly with purified enzyme from human and mouse as well as rabbit by Western blotting (Fig. 4; result not shown for rabbit aldolase). Some other MAb 4E3-reactive peaks eluted with 50 mM-Tris/HCl (pH 7.5)/5 mM-EDTA buffer with little or no aldolase activity also contained M_r 40000 antigen by Western blotting; that could be due to the presence of denatured or inactivated enzyme.

Reaction between MAb 4E3 and aldolase B

In addition to chromatographic and Western blotting analyses, the reaction between MAb 4E3 and aldolase B was examined by e.l.i.s.a. using purified human aldolase B coated onto a microplate (Fig. 6). MAb 4E3 purified by immunoaffinity chromatography was reacted with aldolase B on a microplate (first reaction). Diluted normal human serum was used as a source of complement after the first reaction, and attachment of complement components was detected with peroxidase-labelled anti-C3 antibody (anti-C3 e.l.i.s.a.). As shown in Fig. 6, MAb 4E3 demonstrated strong reactivity, indicating that the complex of aldolase B and 4E3 IgM antibody activated complement component C3. This result further indicated the reactivity between MAb 4E3 and human aldolase B. Similar results were obtained with purified mouse and rabbit aldolase B preparations under identical experimental conditions, supporting the finding that 4E3 recognized the epitope on aldolase B molecule common to human, mouse and rabbit.

DISCUSSION

In the present study, a stable human hybridoma clone, 4E3, has been established by fusing lymphocytes of axillary lymph node taken from a breast cancer patient with human lymphoblastoid cells, HMy2. MAb 4E3 is an IgM_κ antibody and this clone has been producing a large amount of antibody, greater than 10 μg/ml of culture medium, for more than 1 year. MAb 4E3 exhibits binding reactivity against a variety of human normal and cancerous tissues, especially the liver and the kidney; thus it can be designated as an autoantibody and one of the multiple-organ-reactive MAbs (Haspel *et al.*, 1983). The immunochemical and biochemical analyses of the antigen have clearly demonstrated that MAb 4E3 recognizes liver-type aldolase B. Although the possibility cannot be excluded completely at this time that MAb 4E3 may recognize other antigen(s) beside aldolase B, it is interesting and important from biological and clinical points of view to know why such an autoantibody-producing clone has been established in this study.

In spite of extensive investigations for over three decades, the nature or mechanism of autoantibody production is still a controversial subject. Observations such as that autoantibodies are present even in the normal immune system have been reported in contradiction to Burnet's clonal deletion theory (Burnet, 1959). That is, autoantibody can exist in small amounts in a normal host and appear in large quantities when stimulated by factors such as tissue destruction or perturbation of the immune system, even in the absence of antigen stimulation. Guilbert *et al.* (1982) have reported the presence of naturally occurring antibodies against nine common antigens (tubulin, actin, thyroglobulin, myoglobin, ferritin, transferrin, albumin, cytochrome *c* and collagen) in normal human sera, and suggested that natural antibodies directed against a variety of antigens, often self-antigens, can be present in normal sera. Furthermore, Dighiero *et al.* (1983) and Prabhakar *et al.* (1984) have reported the generation of murine hybridomas secreting natural MAbs reacting with self-antigens by using lymphocytes from apparently normal mice. It is intriguing in view of the biological significance of autoantibodies that human and mouse monoclonal autoantibodies thus far obtained rarely react with cell-surface antigens, whereas antibodies reacting with intracellular components or structures occur at a significantly higher frequency (Cote *et al.*, 1983).

With regard to the factors triggering the production of 4E3 antibody in this study, there are several possible explanations. We shall first consider breast cancer. Aldolase B activity in cancer tissue extracts has been found to be generally quite low; and even in the tumour specimens where an appreciable amount of aldolase B is detected, it is well known that the aldolase isoenzyme pattern is shifted from B-dominant (liver or adult type) to A-dominant (muscle or foetal type) in the process of malignant transformation (Hatzfeld *et al.*, 1978). Thus, breast cancer *per se* could be a factor, but may not be the critical one, for stimulation of the aldolase B-sensitized lymphocyte. In this particular patient, two other factors deserve to be discussed; namely, insulin-dependent diabetes mellitus and hepatic disease. In addition to breast cancer, this patient has been suffering from insulin-dependent diabetes for a long time (since 1971). She had an operation for gallstone in 1974, and some enzymes reflecting abnormal liver function (γ-glutamyl transpeptidase and alkaline phosphatase) were slightly elevated at the time (January 1985) when she had surgery for breast cancer. Perturbation in the immunoregulatory system and autoimmune pathogenesis has been strongly suggested to be associated with insulin-dependent diabetes (Rossini *et al.*, 1985); most patients have autoantibodies in their sera especially to endocrine tissues represented by pancreatic islet cells, and they are often suffering from other autoimmune endocrine syndromes including Addison's disease, rheumatoid arthritis and Hashimoto's thyroiditis. Indeed, Satoh *et al.* (1983) have established several autoantibody-producing human MAb clones using peripheral blood lymphocytes from patients with insulin-dependent diabetes.

It is also conceivable that our patient's hepatic disorder may have caused the activation of 4E3 clone. This notion is based upon the following information. A rapid increase in antitissue antibodies has been reported to occur after injection of autologous tissue homogenates, and the induction of acute hepatocellular damage (necrosis) in rats by CCl_4 results in the production of complement-fixing IgM autoantibodies directed against various subcellular components of hepatocytes (Weir, 1963; DeHeer *et al.*, 1974). In part because of these results, a number of authors have postulated that a physiological role of autoantibodies is to facilitate the removal of tissue breakdown products by rendering them recognizable by phagocytes (Elson *et al.*, 1979; Grabar, 1975). The aldolase B level in the serum is known to increase in patients with hepatic disease due to its high content in the liver and is released from damaged tissue (Asaka & Alpert, 1983). Therefore, it is possible that diabetes and hepatic disorder may trigger the production of aldolase B-sensitized lymphocytes in our patient and result in the generation of our 4E3 clone. In an attempt to support this possibility, it is necessary to assay circulating IgM antibody against aldolase B, which we have tried but to no avail. In our experiments using purified aldolase B-coated plates and peroxidase-labelled anti-C3 antibody as detection reagent (anti-C3 e.l.i.s.a.), we observed that such an antibody activity was, if present, very low in the serum specimens of this patient collected during the convalescent state of recovery and was similar to that in sera of normal controls. Unfortunately, no specimens were available to us prior to or at the time of her surgery.

One interesting feature of MAb 4E3 is that it recognizes the antigen with interspecies-common characteristics, as do some autoantibodies (Alvarez *et al.*, 1985; Guilbert *et al.*, 1982). In other words, the molecular structure of antigens recognized by autoantibodies seems to be conserved well during the course of evolution. It may imply some biological significance of autoantibodies. For instance, a similar series of autoantibodies may be present widely in mammalian and other species under the same mechanism of control and exhibiting the same physiological role. Monoclonal autoantibodies, such as 4E3, would be useful probes for these investigations.

As presented in this study, MAb 4E3 reacts with multiple tissues containing aldolase B (especially the liver and the kidney); therefore, it falls into the category of multiple-organ-reactive autoantibodies as proposed by Haspel *et al.* (1983). According to their suggestion, the biological nature of such autoantibodies is of clinical importance, since it provides one possible explanation for the pathogenesis of multiple organ autoimmune disease. The multireactivity of such monoclonal autoantibodies is explained in two ways (Haspel *et al.*, 1983): they react with the same molecule present in several different organs or they react with common antigenic determinants on different molecules in multiple organs. Our results show that MAb 4E3 is a typical example of the former.

It also should be noted that, from technical point of view, accurate measurement of antigen and antigen–antibody complexes is essential for the identification of the antigen molecule recognized by MAb. We have found that the anti-C3 e.l.i.s.a. method employing amplification with complement and peroxidase-labelled anti-C3 antibody as detection reagent is very effective in increasing the sensitivity of assay with IgM-type MAbs (Fig. 6). Therefore, this method can also be used effectively for the assay of trace amount of IgM-type antibody directed against known antigens such as autoantibodies in the sera. In order to elucidate the biological significance of aldolase B and anti-(aldolase B) autoantibody, anti-(aldolase B) antibody activity in normal and various diseases and the mechanism of antibody production should be investigated in future studies.

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